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14. ABSTRACT:
Retinoids and rexinoids are vitamin A derivatives, which cause growth inhibition and/or apoptosis in various cell types, including some breast cancer cells. Retinoids bind and activate the nuclear receptor RAR, whereas rexinoids specifically bind to the related receptor RXR. While retinoids like RA inhibit the growth of estrogen receptor (ER) positive and not ER negative breast cancer cells, rexinoids appear to have activity in both ER positive and ER negative models. In addition, it has been reported that the rexinoid bexarotene can prevent the development of multidrug resistance following exposure to chemotherapy agents. In this report I describe studies of the interactions of different nuclear receptors in breast cancer cells. I have studied how expression of ER affects the phosphorylation status and activity of RARα, and found that reexpression of either ER α or β increases phosphorylation of RARα and also increases the activity of MAPK and PKC pathways. I have also studied how rexinoids modulate the activity of the SXR/RXR heterodimer, and have found that several rexinoids suppress activation of SXR by SXR agonist such as Rifampicin and the chemotherapeutic agent Taxol, which may provide a mechanism to explain how rexinoids can prevent the development of drug resistance.

15. SUBJECT TERMS
Retinoic acid, rexinoids, estrogen receptor, RAR, RXR, SXR, chemotherapy resistance.

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Table of Contents

Introduction

Retinoids are derivatives of vitamin A, which induce differentiation and growth inhibition in a variety of cell types, including breast cancer cells [1-3]. They act mainly by binding to nuclear retinoid receptors, RARs and RXRs, which act as ligand regulated transcription factors [4]. Several natural and synthetic retinoids can inhibit the development of mammary tumors and cause regression of established tumors in rats [5, 6], and there is some clinical evidence that retinoids may be beneficial in breast cancer prevention [7, 8]. Thus, further studies of the potential use of retinoids in therapy of breast cancer are warranted and for this purpose it is important to clarify how retinoids exert their effects on breast cancer cells and what determines sensitivity vs. resistance to these compounds. There is considerable evidence of crosstalk between retinoid signaling and signal transduction pathways activated by growth factors as well as stress stimuli. For example, we have reported that several PKC inhibitors strongly potentiate the response of MDA-MB-231 to RA, and that this involves regulation of PKCδ in particular, as well as *activation* of ERK [9]. Synthetic "rexinoids" (RXR selective retinoid) such as bexarotene (Targretin^{TM}) also have documented antitumor activity in several models of both estrogen receptor (ER) positive and ER negative mammary carcinoma [10-13]. Of note, it has been reported that bexarotene can prevent the development of multidrug resistance following exposure to chemotherapy agents such as Taxol, Cisplatin and Doxorubicin [14-16].

In the first two years of this award, I focused my studies on 1) defining the signal transduction pathways whereby inhibitors of PKC restore sensitivity to retinoids in ER-negative cells and 2) testing the hypothesis that expression of ER alters the phosphorylation status of RAR and/or RXR, thereby rendering the cells sensitive to RA, as described in the approved Statement of Work. Task 1 was fully completed and the results published [9]. Task 2 was partially completed, but the experimental results were less promising. In fact, the hypothesis stated was not supported and my studies of interactions between RAR and ER were not continued. In the third and final year of the award, I have thus refocused my studies on interactions of two other nuclear receptors and their ligands: RXR and SXR (hPXR). SXR is a receptor that is bound and activated by a wide variety of drugs, including Taxol and Cisplatin, and it plays a crucial role in drug metabolism and transport [17-21]. SXR target genes include MDR1, MRP2 and MRP3, as well as several cytochrome P450 enzymes [19, 22-25], and it has been suggested that it may play a role in tumor drug resistance by increasing both the metabolic breakdown and the efflux of drugs like Taxol [19]. Since the transcriptional activity of SXR depends on heterodimerization with RXR [18], I hypothesised that rexinoids may prevent the development of multidrug resistance (see above [14-16]) by interfering with SXR activation by e.g. Taxol.

In this annual report, I will summarize results obtained from studies of the phosphorylation status of RARα. I will also present results showing that rexinoids can suppress activation of SXR by the strong SXR agonist Rifampicin, as well as by Taxol.

Body/Results

1) Stable expression of ERα or β increases basal phosphorylation of RARα.

We had observed that the ER positive and RA sensitive cell line MCF-7 displays less basal phosphorylation of RARα, and also lower PKC activity than the ER negative and RA resistant MDA-MB-231. I thus wanted to assess RARα phosphorylation in MDA-MB-231 stably expressing ER. I used S30 cells, which stably express ERα [26], and a cell line established in our lab, which stably expresses ERβ [27]. The phosphorylation status of RARα was tested by transfecting the cells with Flag-RARα, labeling *in vivo* with ^{32}P-orthophosphate, extracting proteins, and immuno-precipitating the labeled Flag-RARα using anti-Flag-resin (Sigma). Precipitated proteins were separated on a polyacrylamide gel and visualized

using autoradiography. In contrast to the hypothesis, I found that both S30 and ERβ cells displayed *increased* basal phosphorylation of RARα. In addition, these cells displayed slightly enhanced PKC and MAPK activities, compared to the parental MDA-MB-231. Thus, the increased RA-responsiveness seen in these cells [27-29] is *not* dependent on suppression of these pathways, and clearly different from the effect observed with PKC inhibitors, which resensitize both MDA-MB-231 and MDA-MB-468 to the growth inhibitory effects of RA.

2) Rexinoids suppress activation of SXR.

To begin to examine whether rexinoids can modulate the transcriptional activity of SXR, I transiently transfected LS180 colon cancer cells with the reporter plasmid βDR4(3)-tk-luc, containing a consensus SXR response element (SXRE) [18]. LS180 were chosen since they express relatively high levels of SXR and the induction of SXR target genes by rifampicin (a potent SXR agonist) and Taxol has been well characterized in these cells [19, 23, 30]. Four different rexinoids (bexarotene, LGD1305, 9*cis*RA, and LGD268) induced a weak activation of the βDR4 reporter. However, when given in combination with rifampicin, the rexinoids suppressed activation caused by the SXR ligand, indicating an antagonistic function. In contrast, an RAR selective retinoid (TTNPB) neither activated the SXRE reporter, nor suppressed activation by rifampicin. The rexinoids also suppressed rifampicin-induced transcription of CYP3A4-XREM-luc, which contains the proximal and the distal SXREs of the *CYP3A4* promoter [31], indicating a general effect on SXRE containing promoters (data not shown).

It has been reported that breast tumors and cell lines express SXR [32], suggesting that SXR may play a role in chemoresistance in these tumors. I therefore tested the activation of βDR4(3)-tk-luc in MDA-MB-231 and MCF-7 breast cancer cells by bexarotene, rifampicin and Taxol. I found that activation of the reporter by SXR agonists was more potent in MCF-7 than in MDA-MB-231 (10-12 fold *vs*. 2-3 fold by 5μM Rifampicin), and therefore chose MCF-7 as my breast cancer model. These cells were shown to express SXR, but at a lower level than LS180 or HepG2 hepatoma cells (Figure 1). I found that, as in LS180, bexarotene weakly activated transcription in MCF-7 cells, but suppressed activation in response to both rifampicin and Taxol (Figure 2a-b). To confirm that regulation of reporter activity was indeed dependent on SXR, I transfected the cells with βDR4(3)-tk-luc in the presence of either empty pCMX or pCMX-SXR. In the absence of pCMX-SXR, no or very weak activation of this reporter was observed (Figure 2c). No change in renilla luciferase activity produced by the control vector pRL-CMV was seen in response to any of the treatments, indicating lack of non-specific promoter regulation (Figure 2d).

I then tested if bexarotene could suppress the induction of the SXR target gene MDR1/Pgp by rifampicin and Taxol. Western blot analysis of membrane protein fractions showed that both SXR agonists upregulated Pgp in LS180 cells, as previously reported [19, 22]. Further, bexarotene was shown to induce Pgp, but *reduce* the induction by rifampicin and Taxol (Figure 3a), supporting data from transient transfection assays. In MCF-7 cells, flow cytometry was used to determine Pgp levels, since I had difficulty detecting Pgp by Western analysis in these cells. Results confirmed that bexarotene suppressed induction of endogenous MDR1/Pgp by rifampicin (Figure 3b). It is thus feasible that bexarotene, and perhaps other rexinoids, may suppress the induction of drug resistance at least partially by reducing the activation of SXR and the induction of its target genes.

To determine if rexinoid suppression of transcription was associated with reduced binding of SXR/RXR to DNA, gel shift analyses were performed, assessing binding of nuclear proteins to the βDR4 element [33] as well as the ER6 element present in the *CYP3A4* promoter (proximal SXRE [25]). Nuclear extracts from MCF-7 cells treated with rifampicin, bexarotene or both were used. I observed increased binding in the presence of rifampicin, while this effect was reduced by bexarotene (Figure 4 a-b). Supershift analysis with RXR and SXR specific antibodies indicated that both receptors are presents in the major complex binding to the SXRE (not shown). Gel shift analysis using *in vitro* translated proteins confirmed that both receptors were required for binding, and also showed that neither drug affects binding of the heterodimer to the SXRE *in vitro* (Figure 4c). This excludes the possibility of the rexinoid having a direct effect on *e.g.* receptor conformation that reduces its ability to bind the SXRE. Instead, I propose that bexarotene may

cause either a reduction in total receptor levels and/or may induce posttranslational changes that inhibit the ability of SXR and RXR to bind to each other or to transcriptional co-factors needed for optimal activation.

List of Key Research Accomplishments

* Completed the majority of tasks described in the original Statement of Work. Disproved hypothesis stated in Task 2.
* Showed that rexinoids (RXR ligands) can suppress the activation of SXR by the antibiotic rifampicin as well as the chemotherapeutic drug Taxol.
* Showed that bexarotene can suppress induction of the SXR target gene MDR1/Pgp by rifampicin and Taxol.
* Showed that bexarotene reduces the binding of nuclear proteins (SXR and RXR) to SXR response elements.

Reportable outcomes

Abtracts and presentations:

Filippa Pettersson, Nessrine Hanna, Oanh Le, Marina Lagodich, and Wilson, H Miller, Jr. *Rexinoids suppress activation of SXR by SXR agonists rifampicin and paclitaxel in human tumor cells.* Poster, Keystone Symposia, Nuclear Receptors: Orphan Brothers, Banff, March 2006.

Filippa Pettersson, Nessrine Hanna, Marina Lagodich, and Wilson, H Miller, Jr. *Rexinoids Suppress Activation of SXR in Human Tumor Cells: Potential Role in Multidrug Resistance.* Short talk, Canadian Breast Cancer Research Alliance, Reasons for Hope Fourth Scientific Conference, Montreal, May 2006.

Conclusions

During the third and final year of this award, I have completed most of the tasks presented in the original Statement of Work. Notably, experiments performed in relation to Task 2 failed to support the hypothesis stated, and studies were not continued beyond task 2d.
In addition, I made considerable progress elucidating a negative regulation of SXR by ligands of its heterodimer partner RXR, rexinoids. This may partially explain the ability of bexarotene to prevent or reduce the development of drug resistance in tumors treated with chemotherapeutic drugs like Taxol, which are known activators of SXR.

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Appendices

Figures, 2 pages.

APPENDIX: Figures

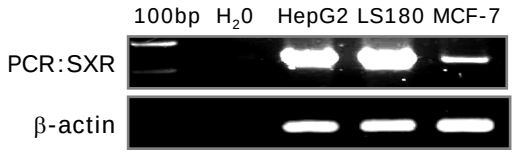

Figure 1. MCF-7 cells express SXR. SXR and β-actin levels were determined by semi-quantitative RT-PCR

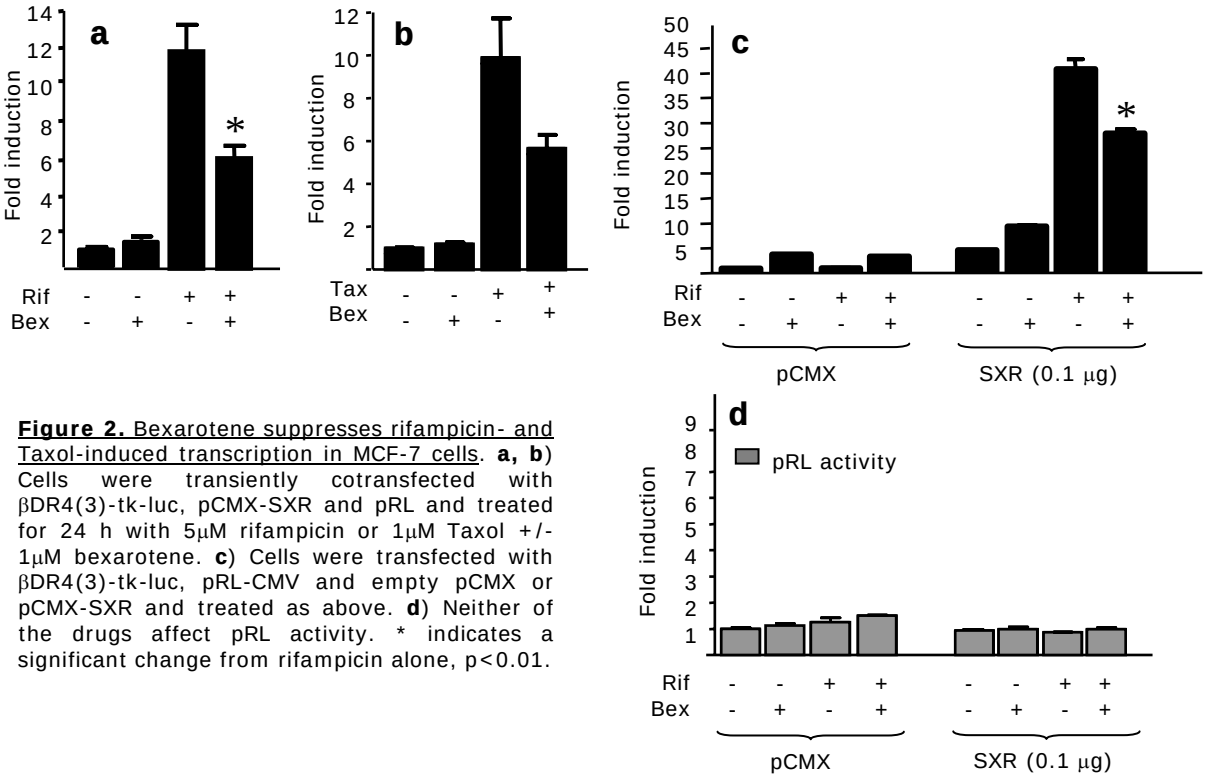

Figure 2. Bexarotene suppresses rifampicin- and Taxol-induced transcription in MCF-7 cells. **a, b**) Cells were transiently cotransfected with βDR4(3)-tk-luc, pCMX-SXR and pRL and treated for 24 h with 5μM rifampicin or 1μM Taxol +/- 1μM bexarotene. **c**) Cells were transfected with βDR4(3)-tk-luc, pRL-CMV and empty pCMX or pCMX-SXR and treated as above. **d**) Neither of the drugs affect pRL activity. * indicates a significant change from rifampicin alone, $p<0.01$.

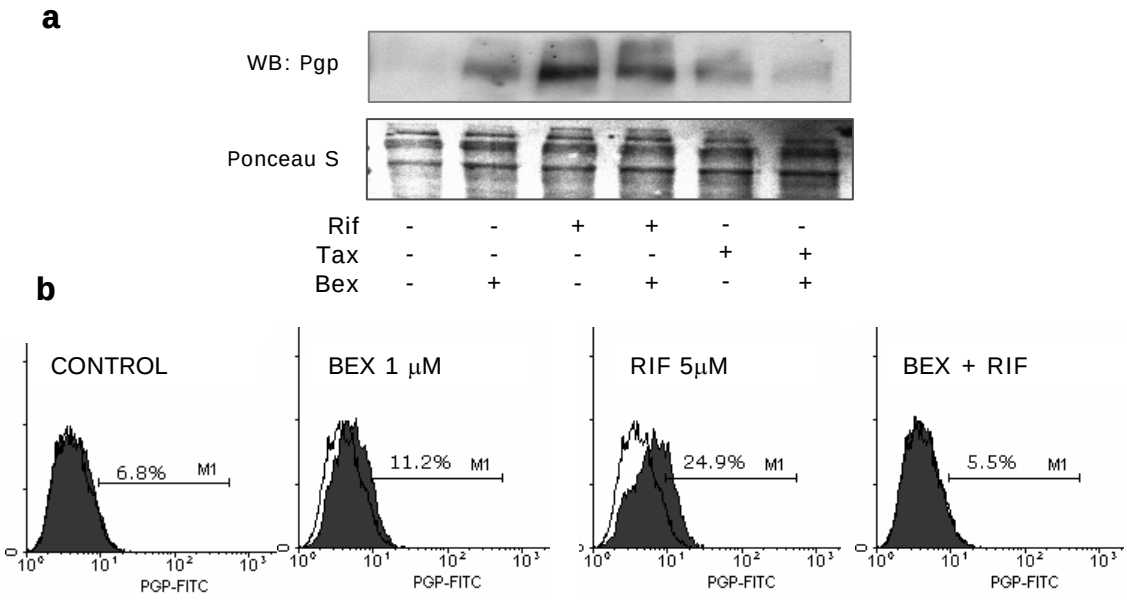

Figure 3. Bexarotene suppresses induction of Pgp. Cells were treated for 48 h with 5μM rifampicin or Taxol and 1μM bexarotene. **a)** Membrane fractions were isolated from LS180 cells and protein levels were analyzed by Western blot. Values indicated are arbitrary units determined by densitometry. As a control for gel loading, Ponceau S staining of the membrane showed equal amounts of total protein. **b)** MCF-7 cells were stained with a FITC labeled Pgp antibody. Pgp positive cells were detected by flow cytometry and numbers shown are percent positive cells compared to the negative control (FITC-mouse IgG, white histogram).

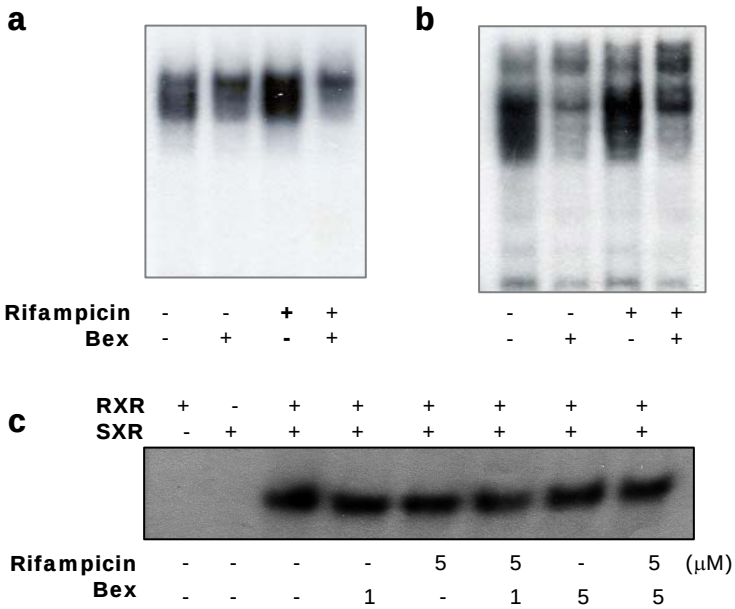

Figure 4. Bexarotene reduces rifampicin-induced binding of nuclear proteins to SXREs. Nuclear extracts from MCF-7, treated as indicated for 24 hours, were incubated with [^{32}P] end-labeled oligonucleotides comprising the βDR4 element (**a**) and the CYP3A4 proximal SXRE element (**b**) and protein/DNA complexes were separated by polyacrylamide gel electrophoresis. **c)** *In vitro* translated SXR and RXR were incubated with [^{32}P] end-labeled *β*DR4 in the presence or absence of the drugs indicated.